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Molecular properties and transcriptional control of the phosphofructokinase and pyruvate kinase genes in a ruminal bacterium, *Streptococcus bovis*

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ABSTRACT

Molecular properties of pyruvate kinase (PYK) and phosphofructokinase (PFK) in *Streptococcus bovis* and transcriptional control of the two enzymes were examined. Sequence analysis indicated that the PYK gene (*pyk*) clusters with the PFK gene (*pfk*) and several other genes. It was demonstrated that the *pyk* and *pfk* are cotranscribed and their transcription appeared to be regulated at the transcriptional level in response to the sugars supplied. The intracellular *pyk*-mRNA level was lower in a catabolite control protein A (CcpA)-disrupted mutant than in its parent strain, and a binding site of CcpA was found in the upstream region of *pfk*. These results suggest that *pfk*–*pyk* transcription is enhanced by CcpA. A recombinant *pyk*-overexpressing strain showed approximately five-fold higher PYK activity, but it did not affect the growth rate or formate-to-lactate ratio significantly, suggesting that the flux in the glycolytic pathway is not altered by an increase in PYK activity.

1. Introduction

Streptococcus bovis is an amylolytic and lactate-producing bacterium, which often predominates in the rumen when ruminants are fed high-concentrate diets [1,2]. Rapid fermentation of starch often leads to an increase in ruminal lactate production by stimulating the growth of amylolytic bacteria such as *S. bovis*, resulting in a drop in ruminal pH. Thus, *S. bovis* may contribute to the progress of lactic rumen acidosis [3], and it is desirable to suppress the overproduction of lactate by *S. bovis*.

Many fermentative anaerobic bacteria gain a major part of their energy through glycolysis, and the rate of glycolytic flux may influence the growth rate and fermentation pattern [4]. Pyruvate kinase (PYK) (EC 2.7.1.40), which converts phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP), has been suggested to control glycolytic flux in some lactic acid bacteria, such as *Lactobacillus* sp. [5,6]. It has also been reported that phosphofructokinase (PFK) (EC 2.7.1.11), which catalyzes the phosphorylation of fructose-6-phosphate to fructose-1, 6-bisphosphate, is a more important control point of the glycolytic flux in some lactic acid bacteria [5,6].

The genes of PFK (*pfk*) and PYK (*pyk*) are clustered and cotranscribed in *Lactobacillus delbrueckii* subsp. *bulgaricus* [7], *Lactococcus lactis* [8], *Streptococcus thermophilus* [9], and *Lactobacillus casei* [10]. In *L. lactis*, expression of the *las* operon, containing *pfk*, *pyk*, and the lactate dehydrogenase (LDH) gene (*ldh*), is involved in the regulation of glycolytic flux [11]. In addition, transcription of the *las* operon and the *pfk*–*pyk* operon, in *L. lactis* [12] and *L. casei* [10], respectively, is regulated by a global transcriptional regulator, catabolite control protein A (CcpA). The transcriptional regulation by CcpA is associated with the PEP-dependent phosphotransferase system (PTS) through HPr protein (heat-stable protein). The CcpA binds to the catabolite-responsive elements (*cre*) that are usually located upstream of, or within, the 5′ region of many operons [13,14].

In *S. bovis*, diauxic growth was observed when *S. bovis* was grown in a medium containing both glucose and lactose [15]. This is due to the fact that glucose is utilized in preference to lactose, although both these sugars are transported through PTS [16,17]. However, diauxic growth disappeared when *ccpA* was disrupted, which indicates that CcpA is involved in catabolite repression in *S. bovis* [15]. The level of CcpA transcription was higher when cells were grown on glucose than when grown on lactose [15]. A *cre* sequence was found in the upper regions of both *ldh* and the gene encoding pyruvate formate-lyase (*pfl*). It was also found that *ldh* transcription is enhanced by CcpA [15], whereas *pfl* transcription is suppressed by CcpA. In addition, the transcription of *gapN*, which encodes $NADP^+$-specific glyceraldehyde-3-phosphate dehydrogenase, is regulated through CcpA [18].

In order to prevent rumen acidosis, it is desirable to control the fermentation in *S. bovis*. From this viewpoint, PFK and PYK may be interesting targets for manipulation as described above. Therefore, we first examined the molecular properties and transcriptional control of *pfk* and *pyk*. We then examined whether *pyk* overexpression affects growth rate and fermentation pattern, because

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the PYK reaction is one of the three ATP-regenerating reactions in *S. bovis* [19], and in addition, PEP is required for PTS [20].

2. Materials and methods

2.1. Sources and growth conditions of S. bovis

The strains used in this study and their characteristics are listed in Table 1. Except where otherwise indicated, the JB1 strain was used. *S. bovis* was anaerobically grown in batch culture as described previously [21]. Briefly, the medium contained (g/L): K_2HPO_4, 0.45; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.9; NaCl, 0.9; $CaCl_2 \cdot 2H_2O$, 0.12; $MgSO_4 \cdot 7H_2O$, 0.19; $Fe(NH_4)_2(SO_4)_2$, 0.1; Trypticase (BBL; Becton Dickinson, Cockeysville, MD), 1.0; yeast extract (Difco Laboratories, Detroit, MI), 1.0; and cysteine HCl, 0.6. As an energy source, either glucose or lactose (3 g/L) was provided. Culture incubations were performed in triplicate, and culture pH was kept between 6.8 and 7.0 [21]. Cell growth was estimated by measuring the optical density at 600 nm. Unless otherwise described, *S. bovis* was grown until the late exponential growth phase. Organic acids were analyzed by high-performance liquid chromatography as described previously [21].

2.2. Sequencing of the pyk and pfk genes

General cloning procedures were as described previously [22,23]. Sequence data were compared with those from other bacteria, and used to identify protein-coding regions as previously described [23]. Two oligonucleotide primers, *pyk*-F1 and *pyk*-R1 (Table 2), designed using the *pyk* sequences registered in the GenBank were used to amplify a part of *S. bovis pyk*. A BLAST search showed that the sequence of the PCR product from the genomic DNA with *pyk*-F1 and *pyk*-R1 (863 bp) had a high degree of identity to the *pyk* sequences from other bacteria. To sequence the regions upstream and downstream from the amplified *pyk* internal region, nested PCR was carried out on a *S. bovis* genomic library [18].

2.3. Analysis of pyk transcription

Total mRNA was extracted as described [22], and RT-PCR was carried out using the total RNA as a template with QIAGEN One Step RT-PCR Kit (Hilden, Germany). The primers used for RT-PCRs (and real-time PCRs) are shown in Table 2. Real-time PCRs were carried out in an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using iQSYBR green supermix (Bio-Rad), according to the manufacturer's recommendations. PCR was carried out under the following conditions. The initial DNA denaturation step at 95 °C for 3 min was followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s and primer extension at 72 °C for 30 s, with a final extension step at 60 °C for 1 min. SYBR green signal measurements were collected for experimental samples in triplicate, and all experiments were performed at least twice. A standard curve was made using seven 10-fold serial dilutions of the PCR products to determine the starting amount for each cDNA template, based on its threshold cycle. cDNA templates were generated from total RNA using iScript cDNA synthesis kit (Bio-Rad). The concentrations of PCR products from chromosomal DNA were estimated by measuring the absorbance at 260 nm, and the concentrations of copy numbers for standard curves were calculated according to the formula described by Yin et al. [24]: copies/mL $= (6.023 \times 10^{23} \times C \times OD_{260})/MWt$, where C is 5×10^{-5} g/mL for DNA and MWt is the molecular weight of PCR product (base pairs $\times 6.58 \times 10^2$ g). When melt curves were run immediately after the last PCR cycle, only one peak was observed each time. Melt curves were constructed by plotting the fluorescence intensities against temperature when the temperature was increased from 60 °C at a rate of 0.4 °C per 10 s (100 cycles). Data were analyzed using the software and graphics programs provided with the iCycler iQ. To confirm the presence and purity, the real-time PCR products were electrophoresed in 1.0% (wt/vol) agarose gel. The copy number of 16S-rRNA was used as an internal standard.

Table 1
S. bovis strains used in this study

Strain	Characteristics	Reference
JB1	Wild type; the parent strain of JB1-*pyk*	[19]
12U1	A highly transformable strain isolated and identified in this laboratory; the parent strain of 12U1-*ccpA*$^-$	[25]
12U1-*ccpA*$^-$	A 12U1 strain having null mutation in *ccpA*	[15]
JB1-*pyk*	The *ldh* promoter region (300 bp upstream of the *ldh* initiation codon) and the *pyk* ORF were integrated into pSBE11, and then introduced into JB1	This study

Table 2
Oligonucleotide primers used in PCR, RT-PCR, and real-time RT-PCR.

Technique	Primer name	Target gene	Sequence (5′–3′)
PCR			
	pyk-F1	*pyk*	TTYAAYTTYTCACAYGG
	pyk-R1	*pyk*	TCDCCHGAMAGCATTGT
RT-PCR			
	RT1	*dnaE*	CGCCGCAAGATTATTG
	RT2	*pfk*	GCCGCCACTAGTCAAA
	RT3	*pfk*	GACATCAACGATGTTGTT
	RT4	*pyk*	ACGGATTTCAGGACCTT
	RT5	*pyk*	ACTGGGGTGTTATTCC
	RT6	*lepB*	CCCTTTTGGCACTTCG
Real-time RT-PCR			
	*pyk*RT-F	*pyk*	TAACGCAGCTGGTAAAG
	*pyk*RT-R	*pyk*	CTGCTTCAACTGGGTA
	pfk RT-F	*pfk*	TCGCTCTTTGGGCAGG
	pfk RT-R	*pfk*	CGATAAGGTGACGTTCT
	*pfk pyk*RT-F	*pfk*	CCACACAAAGCACGTC
	*pfk pyk*RT-R	*pyk*	CAACCGCAGGACCAAG
	Sb16SRT-F	16SrDNA	GAACACCGGTGGCGA
	Sb16SRT-R	16SrDNA	CTCATCGTTTACGGCG

2.4. Preparation of recombinant S. bovis overexpressing PYK

S. bovis pyk open reading frame (ORF) and the promoter sequence of *ldh* were ligated and introduced into pSBE11 as described previously [25]. The recombinant plasmid was electroporated into *S. bovis* JB1, and transformants were selected as described [25]. A transformant harboring the recombinant plasmid was designated as JB1-*pyk* (Table 1). JB1 was used as a control strain, because introduction of the original plasmid did not affect growth and fermentation pattern (unpublished observations).

2.5. Assay for PYK activity

S. bovis cell extracts [21] and recombinant His-tagged proteins of PYK and PFK [25] were prepared as reported previously. PYK and PFK activities were assayed by the methods of Abbe and Yamada [26] and Fordyce et al. [27], respectively. The rate of NADH formation was monitored by measuring the absorbance at